ADENYLATE CYCLASE AND CYCLIC AMP-PHOSPHODIESTERASE ACTIVITIES DURING THE EARLY PHASE OF MATURATION IN *XENOPUS LAEVIS* OOCYTES

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Received 10 November 1980

1. Introduction

Maturation (meiosis) is the transformation, upon hormonal stimulation, of a diploid ovarian oocyte into a haploid and fertilizable egg (review [1]).

In amphibians, in vitro maturation of oocytes dissected out of the ovary can generally be induced by a short incubation of the oocytes with progesterone. In *Xenopus laevis*, the process of maturation extends over 6–10 h; it involves several different steps the most important of which, from a biological viewpoint, is the appearance of the maturation promoting factor (MPF) after 4–6 h hormonal treatment [2].

An essential step in the initiation of maturation is the dephosphorylation of certain classes of proteins. This phenomenon is believed to result from the following sequence of events: an intracellular release of membrane-bound Ca^{2+} would be instantaneously triggered by progesterone; Ca^{2+} and calmodulin would activate a cyclic AMP (cAMP)-phosphodiesterase. A drop of cAMP content would follow; as a result, a cAMP-dependent protein kinase activity would decrease with a subsequent dephosphorylation of proteins.

Some of these steps are well substantiated by experimental data, whereas others are still hypothetical: it has been established that progesterone treatment induces the release of Ca^{2+} from the membrane within 30–90 s [3]; a calmodulin-like protein has been isolated from *Xenopus* oocytes [4]; moreover, Ca^{2+}–calmodulin injected into oocytes has been reported to induce their maturation [5], but it has not yet been proved that Ca^{2+} and calmodulin activate in vivo an oocytic cAMP-phosphodiesterase. However, in [6] protein dephosphorylation is clearly indicated as an essential event for the initiation of maturation. Injection into the oocytes of either highly purified preparations of the regulatory (R) subunit of the cAMP-dependent protein kinase or of the thermostable protein kinase inhibitor (I) both induce maturation. However, injection of the catalytic (C) subunit inhibits progesterone-induced maturation. The inhibition of a cAMP-dependent kinase should result from a drop in cAMP content of the oocyte. However, the results obtained on the cAMP content in *Xenopus laevis* oocytes are discrepant: apparently, a drop in cAMP content occurs either at the very beginning (30 s) of progesterone treatment [7], or after 3 h [8], or even does not occur at all [9–11].

A change in cAMP level may be induced by changes in activities of either adenylate cyclase or phosphodiesterase which, respectively, control the rate of cAMP synthesis and degradation. It has been shown [12] that adenylate cyclase is inhibited after a 1 h treatment with progesterone, but nothing is known about the behavior of the enzyme at earlier stages in the process of maturation. On the other hand, phosphodiesterase has been measured in vitro after a 1 h progesterone treatment and its activity was not modified [13].

Owing to the almost instantaneous release of membrane Ca^{2+} [3], and to the possible fast effect of progesterone on cAMP content of the oocytes [7], we have investigated the in vivo behaviour of phosphodiesterase and adenylate cyclase during the first minutes of progesterone treatment.

For adenylate cyclase determinations, we have worked out experimental conditions allowing a sufficient protection of the neosynthesized labelled cAMP without impairing maturation as is the case with phosphodiesterase inhibitors such as isobutylmethylxanthine or theophyllin.

We report here that progesterone treatment does not modify the activity of phosphodiesterase at least during the first 10 min; in contrast, adenylate cyclase activity is inhibited by progesterone, but only right at the beginning of the hormonal treatment.

2. Material and methods

Female *Xenopus laevis* were obtained from a dealer. Full-grown oocytes (stage VI, [14]) were isolated by manual dissection with watchmaker's forceps and maintained in modified Barth's solution (MBS) [15] without antibiotics. Germinal vesicle breakdown (GVBD) in oocytes treated with progesterone (10 μg/ml) was followed by observing the appearance of a white spot at the animal pole, and ascertained by dissection of the boiled oocytes.

In enzymatic determinations, each point of the kinetic studies represents the mean of 5 determinations made on groups of 5 oocytes each (SEM indicated in the figures). The injection of 5 oocytes takes at most 30 s.

2.1. *Phosphodiesterase activity*

c[^{3}H]AMP (0.3 ng = 10 000 cpm) 50 nl, were injected into each oocyte. At the indicated times, the oocytes were placed in ice-cold 1 M PCA, homogenized and centrifuged at 3000 × *g* for 10 min. The supernatant was purified over an alumina column (1 g), as in [16] and then over a 2 ml Dowex AG 1-X2 column as follows: after loading the Dowex column with the formate eluate from the alumina column, adenosine was eliminated with 10 ml 0.5 mM HCl. cAMP was subsequently recovered by eluting the column with 3 ml 0.5 M HCl ([17]; M. G., unpublished). After addition of 6 ml Aquasol-2 NEN, the eluates were counted in a liquid scintillator. The recovery from the column purification was measured by addition of a known amount of c[^{3}H]AMP to a PCA extract of oocytes, and reproducibly found to be ~85%.

2.2. *Adenylate cyclase activity*

Each oocyte was injected with 50 nl 0.1 M MES (pH 6.5) containing 700 000 cpm of [α-^{32}P]ATP. c[^{32}P]AMP was extracted, purified and measured as above.

3. Results and discussion

3.1. *Determination of cAMP phosphodiesterase activity*

3.2.1. Effect of progesterone

After the injection of tracer c[^{3}H]AMP, the oocytes were immediately transferred into either plain or progesterone-containing MBS. The fate of c[^{3}H]AMP was followed during the first 10 min after its injection (fig.1).

Fig.1 shows that the activity of phosphodiesterase measured in vivo in the oocytes is linear during at least 10 min. During this period, progesterone does not modify the activity of the enzyme at all.

It should be pointed out that the injected tracer c[^{3}H]AMP (0.3 ng) only dilutes the endogenous pool (1.0 ng) of the oocyte slightly. We thus consider that our phosphodiesterase determinations reflect better the true in vivo situation than estimations made in the presence of a huge excess of injected cAMP [8], which might artificially enhance the activity of the enzyme.

3.2. *Determination of adenylate cyclase activity*

3.2.1. Conditions for in vivo determination

We have designed conditions to protect the cAMP neosynthesized from [α-^{32}P]ATP during in vivo adenylate cyclase determinations. Injection of a large excess of unlabelled cAMP into the oocytes allowed a

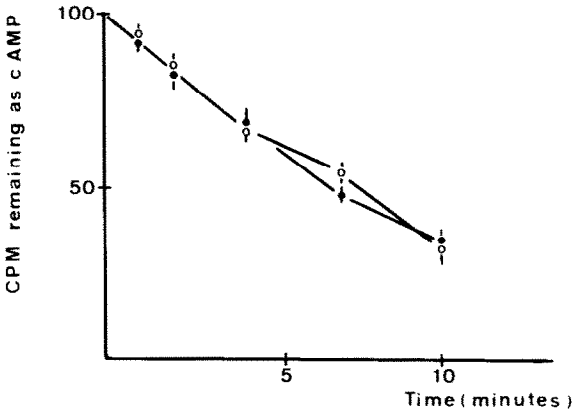

Fig.1. Determination of phosphodiesterase activity in oocytes injected with ±10 000 cpm each of c[^{3}H]AMP (0.3 ng) and immediately placed in MBS (•) or MBS containing 10 μg/ml progesterone (○). At different times after the injection, the remaining c[^{3}H]AMP was extracted and purified as in section 2. Each determination was performed on 5 batches of 5 oocytes.

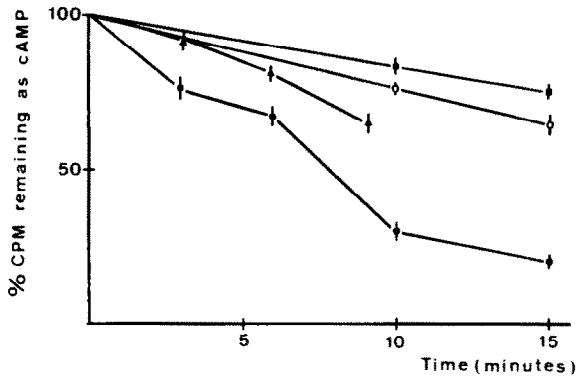

Fig.2. Effect of an excess of injected cold cAMP on the in vivo hydrolysis of tracer c[^{3}H]AMP. Each oocyte received 50 nl 0.1 M MES buffer (pH 6.5) with either ±10 000 cpm (0.3 ng) of c[^{3}H]AMP alone (•) or the same containing in addition 50 ng (○) or 100 ng (■) of unlabelled cAMP. Other oocytes (▲) were preincubated for 3 h with 1.0 mM IBMX before the injection of c[^{3}H]AMP. At the indicated times after the injection, the remaining c[^{3}H]AMP was extracted and purified as in section 2. Each determination was performed on 5 batches of 5 oocytes.

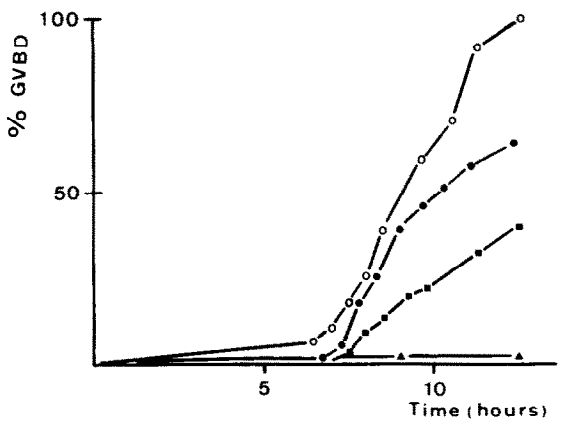

Fig.3. Effect of injected cAMP on the rate of maturation (GVBD). Oocytes (30) were injected with 50 nl each of 0.1 M MES buffer (pH 6.5) either alone (○) or containing 50 ng cAMP (•) or 100 ng cAMP (■). Immediately after the injection, the oocytes were placed in MBS containing 10 μg progesterone/ml. Some oocytes (▲) were preincubated for 3 h in 1 mM IBMX in MBS, then transferred into a progesterone solution. The percentage of GVBD was scored at different times of progesterone treatment.

substantial protection of the tracer c[^{3}H]AMP (fig.2). cAMP at 50 or 100 ng/oocyte (i.e., 50- or 100-times their endogenous content) respectively, inhibit by 67% and 77% the disappearance of c[^{3}H]AMP during the 10 min following injection.

We have also studied the protective effect of isobutyl-methyl-xanthine (IBMX) reported [12] to totally inhibit the in vitro activity of phosphodiesterase in *Xenopus laevis* oocytes. We show here that this methylxanthine slows down the activity of phosphodiesterase by only 50% in 10 min when measured in vivo (fig.2).

It can be seen that preincubation in IBMX as well as injection of 50 ng cAMP ensures a good protection of a tracer amount of c[^{3}H]AMP injected into the oocytes. However, increasing the intracellular content of cAMP seems to be preferable: as illustrated in fig.3, IBMX totally impairs progesterone-induced maturation, as shown in [18], whereas an excess of cAMP only slightly delays GVBD.

3.2.2. Effect of progesterone

The above results led us to carry out the in vivo assay of adenylate cyclase activity by injecting into each oocyte 50 ng unlabelled cAMP together with [α-^{32}P]ATP. Under these conditions, we first observed (not shown) that the amount of c[^{32}P]AMP recovered remains linear for only 4 min after the injection of [α-^{32}P]ATP. At later times, the increase in the c[^{32}P]-AMP recovered was no longer linear with time. The observed plateau must result from many conflicting enzymatic activities (ATPase, phosphodiesterases).

Fig.4 illustrates the behaviour of adenylate cyclase

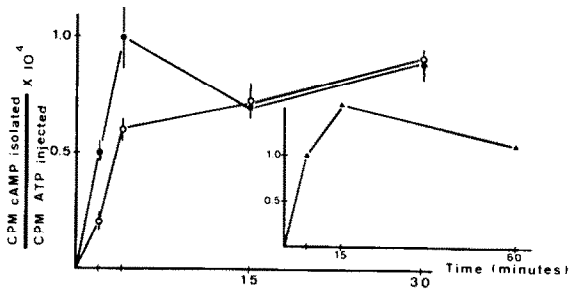

Fig.4. Determination of adenylate cyclase activity in oocytes injected with 50 nl each of 0.1 M MES buffer (pH 6.5) containing 50 ng unlabelled cAMP and ±700 000 cpm of [α-^{32}P]-ATP. Immediately after the injection, the oocytes were placed in MBS (•) or MBS containing 10 μg progesterone/ml (○). Other oocytes (▲) were preincubated for 3 h in MBS containing 1 mM IBMX, then injected with 50 nl 0.1 M MES buffer (pH 6.5) containing ±700 000 cpm of [α-^{32}P]ATP and maintained in the IBMX solution for 1 h (inset). At the indicated times after the injection of [α-^{32}P]ATP, cAMP was extracted and purified as in section 2. Adenylate cyclase activity was expressed as the ratio of ^{32}P counts found in cAMP over the ^{32}P counts in the injected ATP × 10^4. Each determination was performed on 5 batches of 5 oocytes.

in control oocytes and following hormonal treatment. It shows that progesterone inhibits the activity of adenylate cyclase: only 40% and 60% of the c[^{32}P]-AMP found in the controls were recovered after 2 and 4 min progesterone treatment, respectively. This inhibition is transient, since after 15 min the amount of c[^{32}P]AMP is the same in progesterone-treated and in control oocytes.

The inset shows that even after a 3 h pretreatment with IBMX, the activity of adenylate cyclase ceases to be linear already after 6 min; this result was to be expected since we have shown (fig.2) that the oocyte's phosphodiesterase is only partially inhibited by IBMX in vivo. Therefore, even in the presence of IBMX, the only reliable data on adenylate cyclase activity in oocytes are those recorded in the first 5 min after injection of [α-^{32}P]ATP; this makes questionable the physiological meaning of the cyclase activities measured 1 h after the injection of the radioactive substrate [12].

The early and transient decrease in adenylate cyclase activity reported here is consistent with [7] where cAMP content of the oocyte dropped by 40% during the first 60 s progesterone treatment, then returned to its basal level after 10 min.

It is likely that the increase in Ca^{2+} temporarily released from the cortex as a primary response to progesterone induces, an inhibition of adenylate cyclase rather than an activation of cAMP phosphodiesterase, perhaps via a Ca^{2+}–calmodulin complex. Indeed, a biphasic response of adenylate cyclase to changes in [Ca^{2+}] has been described in brain [19] where the activity of the enzyme is slowed down in the presence of an excess of Ca^{2+}.

Our results substantiate the hypothesis that a drop of cAMP is a prerequisite for the release of the meiotic block. It has been shown that cholera toxin, which irreversibly stimulates adenylate cyclase in animal cells [20], is an inhibitor of progesterone-induced maturation [21]. Since we have shown [22] that an injection of GTP in the oocytes also inhibits progesterone-induced maturation, it was expected to find an activation of adenylate cyclase in the injected oocytes, by analogy with the action of cholera toxin. But it seems from preliminary results obtained in our laboratory that GTP does not activate an adenylate cyclase in the oocytes and that the inhibition of progesterone-induced maturation is to be ascribed to a rather unexpected inhibition of cAMP phosphodiesterase, which is reminiscent of the effects of IBMX [18].

Acknowledgements

We thank Professor R. Kram for helpful suggestions and Professor J. Brachet who kindly accepted to discuss the manuscript. This work was supported by the Fonds de la Recherche Fondamentale Collective; E. B. is Maître de Recherches du Fonds National de la Recherche Scientifique; J. H.-Q. is Chercheur Qualifié du Fonds National de la Recherche Scientifique.

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